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EXHIBIT A

ASSESSMENT OF TOPICAL ANTI-INFLAMMATORY ACTIVITY IN RATS WITH CANTHARIDIN-INDUCED INFLAMMATION

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Topical application of 400 μg of cantharidin to the rat's ear caused an approximate doubling in the mean weight of uniform ear punch samples when compared to vehicle-treated controls at 72 hr, and produced a maximal response at 7 days. Dexamethasone reduced the increase in weight when applied topically, but was ineffective when given subcutaneously or orally at the same doses.

Hydrocortisone, prednisolone, triamcinolone, betamethasone, fluorometholone, parametha-sone acetate, fluocinolone acetonide, fluocinonide, and flurandrenolide showed significant suppression of cantharidin-induced inflammation. Cholesterol, diphenhydramine, tripelen-namine, chlorpheniramine, promethazine, cyproheptadine, epinephrine, phenylephrine, alpha-tocopherol, indomethacin, and bufexamac were inactive. It is suggested that the procedure employed may be useful in the screening and evaluation of topical anti-inflamma-tory agents.

Various procedures are available for the assessment of topical anti-inflammatory activity in man. These techniques, developed for investigation of the topical effectiveness of corticosteroids, include methods based upon inhibition of inflammation induced with various chemical irritants such as mustard oil and nitric acid [1], croton oil [2], and tetrahydrofurfuryl alcohol [3]. Other procedures take advantage of the blanching or vasoconstrictor property of topically applied corticosteroids [4], since tests of various corticosteroids have shown good correlation between vasoconstrictor potency and topical effectiveness clinically in inflammatory skin conditions [5–7].

Animal models suitable for finding and evaluating topically active, anti-inflammatory compounds are not nearly as numerous as their clinical counterparts. Several animal methods are based on the "local" effect of compounds applied to cotton pellets implanted subcutaneously [8], or injected directly into granuloma pouches induced with croton oil [9,10]. It is questionable whether such "local" effects mimic topical activity and are predictive of dermatologic effectiveness.

A direct approach to the determination of topical anti-inflammatory activity in an animal model is the method developed by Tonelli and his associates [11]. This procedure is based on the inhibition of rat ear inflammation induced with croton oil. Several variations of this method have been described [12–14]. These reports would seem to indicate widespread application of this procedure by pharmaceutical laboratories. In our experience, the response to croton oil, applied topically or in granuloma pouch procedures, has been quite variable. This is perhaps not unexpected, since croton oil, a relatively crude mixture of many constituents, varies in its irritant properties in different batches and with aging.

In this report we shall present data in rats on inflammation induced by the topical application of cantharidin. The results obtained demonstrate that cantharidin produces an inflammation which is amenable to topical corticosteroid mitigation, and that the procedure employed may be a useful method for finding and evaluating topical anti-inflammatory compounds.

MATERIALS AND METHODS

Charles River CD 21- to 22-day-old male rats, 50 to 60 gm body weight, were given an intraperitoneal injection of 0.12 ml Chloropent (Fort Dodge Laboratories, Fort Dodge, Iowa 50501). After the animals were anesthetized, 0.1 ml of the irritant solution was applied topically to the outer surface of one ear with a 0.5-ml hypodermic syringe fitted with a 1"-long 22-guage needle. The use of anesthetized animals enabled the accurate application of the topical solutions, and prevented the animals from rubbing off the material prior to drying. The rats remained anesthetized for approximately 3 hr. A standardized vehicle was employed for all experiments and consisted of a mixture of 1 part ethanol, 1.5 parts collodion (USP), 2 parts acetone, and 3 parts anhydrous diethyl ether by volume. Cantharidin BP 1949 (J.H. Walker and Co., 22 W. First St., Mount Vernon, N.Y. 10550) alone or together with test compound was dissolved in this vehicle. Separate aggregates or rats were employed as vehicle controls, cantharidin-alone group, and cantharidin plus test compound group(s), and only one ear per rat was employed.

In all experiments, except the time course study (see Fig. 2), animals were autopsied 72 hr after topical application of cantharidin solutions. The rats were killed in CO_2 chambers and positioned so that the treated ears

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were lying flat on a cork board covered with Parafilm M (American Can Co., New York, N.Y.). Samples of each treated ear were obtained by punching out a uniform disc with a #3 cork borer (9/32" diameter) and weighing each tissue sample to the nearest 0.1 mg. Means, standard errors, and statistical significance by Student's *t*-test were computed in the usual manner.

RESULTS AND DISCUSSION

The effect of the dose of topically applied cantharidin on ear punch weight measured 72 hr after application is shown in Figure 1. Topical doses of 50 μg or more increased ear punch weight significantly above vehicle-treated controls. The response appeared to follow a linear log-dose relationship, and apparently attained the maximal response level with a dose at or near the 400-μg level, since doses of 800 or 1000 μg failed to cause further increments in response. The response to 400 μg of cantharidin was very consistent. In a series of 20 separate experiments performed dur-

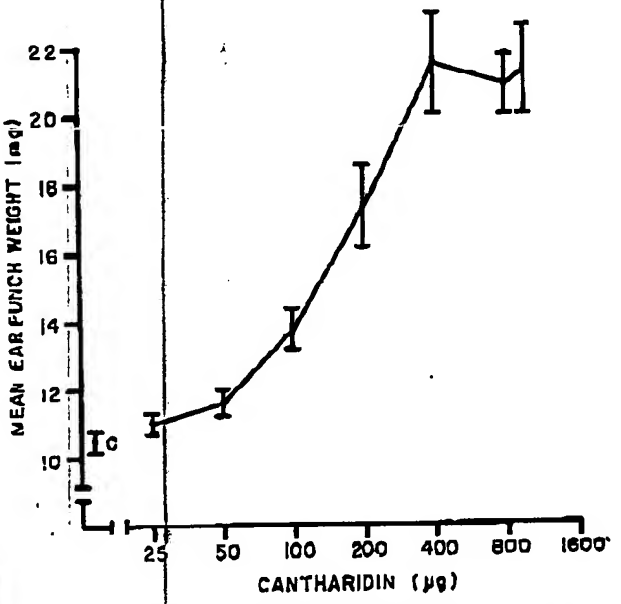


FIG. 1. Effect of cantharidin dose on rat ear punch weight at 72 hr. Nine to 10 rats per group. *Vertical lines* represent standard errors of means. *C* = vehicle-treated controls.

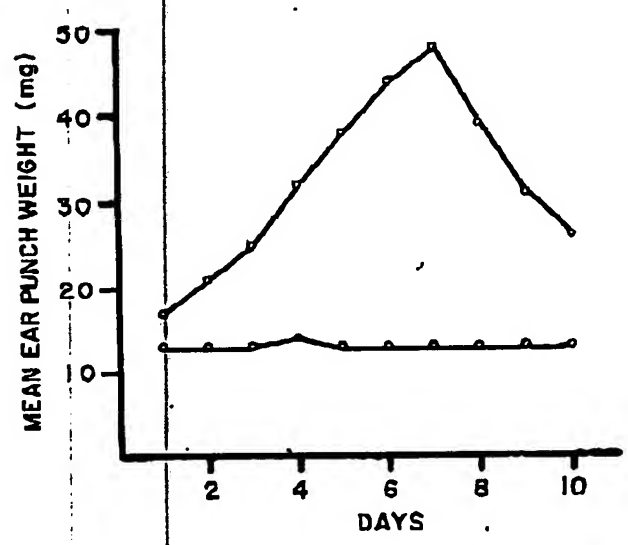
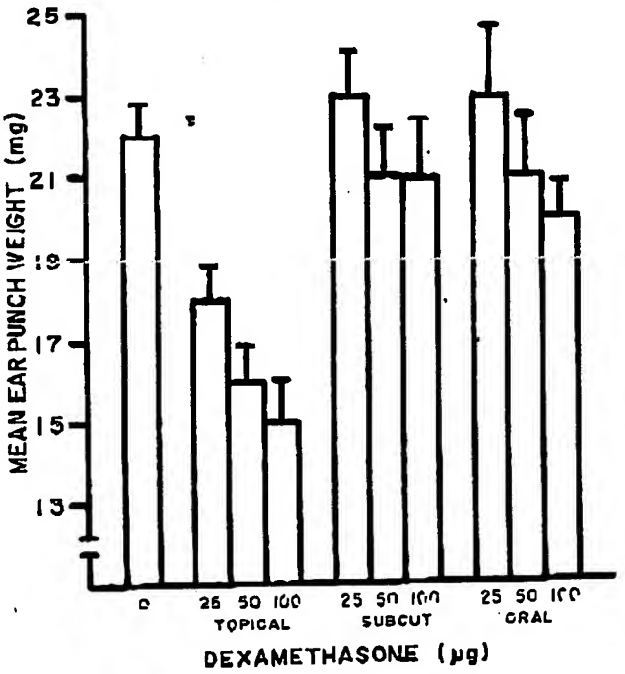


FIG. 2. Time-course of topical inflammation by 400 μg of cantharidin. Each point represents the mean of 10 rats. Rang of standard errors: vehicle controls (*lower curve*) 0.1–0.9, cantharidin-treated (*upper curve*) 0.3–1.9. Differences between means at all time points significant at $p < 0.001$.



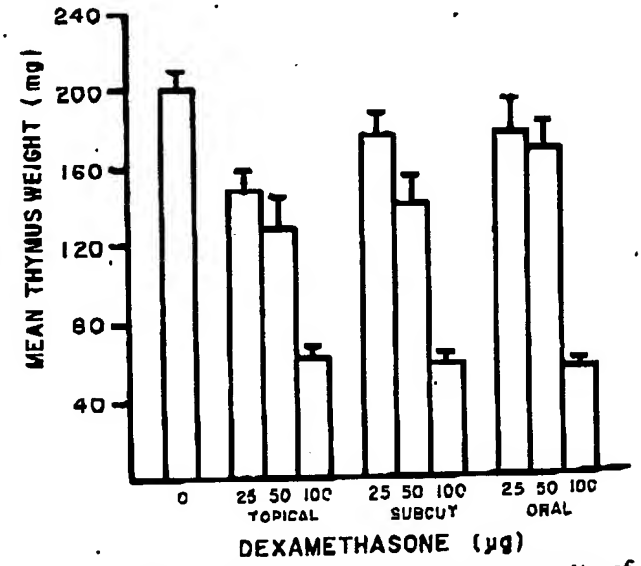


FIG. 3. Effect of administration route on activity of dexamethasone. *a*: Ear punch weight. All rats treated topically with 400 μg cantharidin with autopsy at 72 hr. Irritant-alone group had 20 rats. All other groups had 7 to 10 rats. Only topical dexamethasone groups significantly different ($p < 0.01$) from cantharidin-alone group. Oral and subcutaneous vehicle was 10% ethanol/90% sesame oil by volume. *b*: Thymus weight. Same rats as in Fig. 3*a*. All mean thymus weight differences from cantharidin-alone group significant ($p < 0.01$) except dexamethasone (subcutaneous) at 25 μg dose, and dexamethasone (oral) at 25 and 50 μg doses.

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Figures 3a

TABLE. *Effects of various corticosteroids and some nonsteroid compounds on topical inflammation induced with cantharidin*

All rats had 400 μg of cantharidin applied topically 72 hr before autopsy. Each group had 10 rats unless indicated otherwise by value in parenthesis. Differences between − Compound and + Comp und groups not significant statistically for any nonsteroid compound. Percent inhibition based on increase from vehicle controls.

Compound	Topical dose (μg)	Mean ear punch weight (mg) ± SE		Significance of difference	Percent inhibition
		− Compound	+ Compound		
Hydrocortisone	320	26.2 ± 0.9 (20)	20.1 ± 0.7 (19)	$p < 0.001$	52
Prednisolone	80	23.3 ± 0.7	19.2 ± 1.3 (9)	$p < 0.001$	35
Triamcinolone	100	26.8 ± 0.8 (19)	22.2 ± 0.8 (19)	$p < 0.001$	31
Betamethasone	40	20.4 ± 0.6 (12)	17.4 ± 1.0 (9)	$p < 0.05$	32
Dexamethasone	40	19.9 ± 1.0	14.6 ± 1.1	$p < 0.01$	63
Fluorometholone	50	21.1 ± 1.5 (17)	17.0 ± 0.9 (17)	$p < 0.05$	40
Paramethasone acetate	100	21.1 ± 1.5 (17)	16.8 ± 0.6 (17)	$p < 0.05$	41
Fluocinolone acetonide	20	21.0 ± 1.2 (12)	14.3 ± 0.4 (12)	$p < 0.001$	67
Fluocinonide	10	24.5 ± 1.9 (9)	17.7 ± 1.2	$p < 0.01$	53
Flurandrenolide	40	20.4 ± 1.3 (12)	15.7 ± 1.3 (12)	$p < 0.05$	52
Diphenhydramine[a]	1000	20.0 ± 1.3	18.5 ± 0.8		
Tripelennamine	8000	20.9 ± 1.0	22.7 ± 1.0		
Chlorpheniramine[b]	1000	18.8 ± 0.6	19.5 ± 1.1		
Promethazine[a]	1500	22.6 ± 0.7	21.7 ± 1.2		
Cyroheptadine[a]	1000	21.1 ± 0.5	22.6 ± 1.4		
L-Epinephrine	4	18.8 ± 0.6	17.9 ± 1.2		
L-Phenylephrine[a]	250	22.6 ± 0.7	21.8 ± 1.7		
DL-α-Tocopherol	8000	23.1 ± 1.2	22.7 ± 1.1		
Indomethacin	1600	17.2 ± 1.1	18.9 ± 1.2		
Bufexamac	1200	20.9 ± 1.5	18.8 ± 1.0		

[a] Hydrochloride
[b] Maleate

ing a 1-year interval, the increase in ear punch weight above controls varied from 79 to 106% with an average increase of 88%. These data demonstrate that the inflammatory response to topically applied cantharidin, as measured by difference from control in tissue sample weight, is highly reproducible and consistent quantitatively.

The course of topical inflammation induced with cantharidin is shown in Figure 2. Inflammation was clearly evident 1 day after topical application of 400 μg of cantharidin, and attained its maximal degree at 7 days. The induced inflammation, as measured by tissue weight changes, subsided after 7 days, but ear punch weights were still not at control levels at 10 days.

It was considered of interest to determine whether an inactive steroid would mitigate cantharidin inflammation. Tests with cholesterol showed that it was ineffective in suppression of cantharidin inflammation at concentrations up to 10%. Since most topically effective corticosteroids are active at concentrations considerably less than 1%, the ability to inhibit cantharidin inflammation at reasonable dosages would easily distinguish active from inactive steroids. Indeed, as shown in Figure 3*a*, dexamethasone was effective topically at a concentration of 0.025% or less.

It was of interest to determine whether inhibition of cantharidin-induced inflammation by topically applied corticosteroids was due to systemic absorption or direct effect at the site of application. Figures 3*a* and *b* show the results obtained on ear punch weights and thymus gland weights after topical, subcutaneous, and oral administration of dexamethasone. Since thymus involution occurred after topical application of dexamethasone, it is evident that systemic absorption occurred. However, although subcutaneous and oral doses of the steroid caused equal thymus effect, only topically applied dexamethasone showed significant reduction in ear punch weights. These data demonstrate that the anti-inflammatory activity resulted from direct effect on the skin.

In the Table are presented typical responses obtained in this test procedure with various corticosteroids. Although only single dose–response data are reported for each compound, the degree of response at the dose employed gives some indication of relative effectiveness. In general, relative activity appeared to correlate with clinical effectiveness. In other studies, we have obtained linear log–dose response curves for every topically activ corticosteroid that has been examined by this procedure.

In the Table data are also presented on some nonsteroidal compounds which have been tested for topical activity against cantharidin-induced inflammation. Several antihistamines (diphenhydramine, tripelennamine, chlorpheniramine, promethazine), a serotonin and histamine antagonist (cyproheptadine), and two vasoconstrictors (epinephrine, phenylephrine) were without effect at the dosages tested. Vitamin E has been reported to have both systemic and topical anti-inflammatory

activity [15]. As shown in the Table, DL-α-tocopherol was ineffective in suppression of cantharidin-induced inflammation when applied topically in an 8% solution. Indomethacin, which is active in croton oil-induced inflammation when applied topically [14], was ineffective against cantharidin inflammation. Bufexamac has been reported to be effective topically in ultraviolet erythema and against carrageenin-induced cutaneous edema [16]. Against cantharidin inflammation, bufexamac was inactive at a topical dose of 1.2 mg (1.2% solution).

Since the characteristics of the inflammatory response to cantharidin, its possible mitigation by drugs, its reproducibility and statistical variation were unknown when these studies were initiated, it was considered desirable to include vehicle controls in each experiment. This enabled monitoring of the consistency of the response to cantharidin from experiment to experiment. Rats were not housed in individual cages, but in treatment groups, consequently, the use of only one ear per animal prevented physical contact by drug-treated ears with ears treated with irritant alone. It is obvious that the procedure can be modified, and perhaps improved, by using both ears and allowing each rat to serve as its own control.

Selection of different observation times may also be indicated for compounds with different bioavailability characteristics. It is possible that the nonsteroid compounds tested might have shown topical anti-inflammatory effects if observations had been made at other time points or with higher dosages. It should be noted, however, that all of the steroids tested showed significant inhibition of inflammation at 72 hr, and were effective at relatively low doses. The 72-hr observation period was selected because it gave a significant degree of inflammation (approximately 90% above vehicle controls) from which to measure drug effects after only a single application of drug. Presumably, measurements at peak response (7 days) would require multiple drug applications, or high drug concentrations to give drug effects quantitatively equal to those obtainable at the 72-hr time period. From a practical standpoint, the use of minimal quantities of test compounds for screening or study is advantageous, especially with compounds that are difficult and/or expensive to synthesize.

Cantharidin-induced inflammation appears to be a useful procedure for screening and evaluation of topically active, anti-inflammatory compounds. The procedure would seem to be especially useful for the assessment of the activities of topical corticosteroids. The data obtained with nonsteroids would suggest that its value for testing such weaker compounds may be somewhat less, since indomethacin and bufexamac, agents active topically in other test procedures, were ineffective against cantharidin-induced inflammation. However, the limited experience with nonsteroids, and the relative paucity of topically effective, nonsteroidal, anti-inflammatory compounds currently availabl for testing, preclude a definitive judgement in this regard.

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REFERENCES

1. Scott A, Kalz F: The effect of the topical application of corticotrophin, hydrocortisone, and fluocortisone on the process of cutaneous inflammation. J Invest Dermatol 26:361–378, 1956
2. Witkowski JA, Kligman AM: A screening test for anti-inflammatory activity using human skin. J Invest Dermatol 32:481–483, 1959
3. Schlagel CA, Northam JI: Comparative anti-inflammatory efficacy of topically applied steroids on human skin. Proc Soc Exp Biol Med 101:629–632, 1959
4. McKenzie AW, Stoughton RB: Method for comparing percutaneous absorption of steroids. Arch Dermatol 86:608–610, 1962
5. McKenzie AW: Percutaneous absorption of steroids. Arch Dermatol 86:611–614, 1962
6. McKenzie AW, Atkinson RM: Topical activities of betamethasone esters in man. Arch Dermatol 89:741–746, 1964
7. Reid J, Brookes DB: Topical corticosteroids—an experimental evaluation of the vasoconstrictor test as an index of anti-inflammatory activity. Br J Dermatol 80:328–336, 1968
8. Hershberger LG, Calhoun DW: A cotton pellet granuloma assay for locally administered anti-inflammatory drugs: 9-halo,21-desoxy-corticoids. Endocrinology 60:153–160, 1957
9. Selye H: Use of "granuloma pouch" technic in the study of antiphlogistic corticoids. Proc Soc Exp Biol Med 82:328–333, 1953
10. Robert A, Nezamis JE: The granuloma pouch as a routine assay for antiphlogistic compounds. Acta Endocrinol 25:105–112, 1957
11. Tonelli G, Thiabault L, Ringler I: A bio-assay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied corticoids. Endocrinology 77:625–634, 1965
12. Riesterer L, Jaques R: Anti-inflammatory action of a benzylglucofuranoside applied topically. Experientia 24:581–582, 1968
13. Dorfman RI: Biological activity of topical corticoids. Br J Dermatol 82[suppl 6]:45–48, 1970
14. VanArman CG: Anti-inflammatory drugs. Clin Pharmacol Exp Ther 16:900–904, 1974
15. Kamimura M: Antiinflammatory activity of vitamin E. J Vitaminol (Kyoto) 18:204–209, 1972
16. Lambelin G, Vassart-Thys D, Roba J: Pharmacological studies of bufexamac topically applied on the skin. Arch Int Pharmacodyn Ther 187:401–414, 1970

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